

Session 2A: Metrics for Universal Standards: Expression Arrays

Producing Quality RNA Samples and Standards - an RNA Reagent Company Perspective

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Universal RNA Standards Workshop, March 28-29, 2003

Ambion Inc.

- **Sample collection**
- **RNA extraction**
- **RNA preservation and storage**
- **Enzymatic manipulations of RNA**
 - Reverse Transcription
 - RTPCR
 - RNase treatment
 - DNase treatment
- **Detection and Analysis of specific RNAs**
 - RPA, Northern blot, Real-time PCR
- **Gene Expression analysis with microarrays**
 - RNA Amplification with MessageAmp
 - Control RNA spikes
- **RNase Control**
- **Production of RNA**
 - By biological samples (total RNA and mRNA)
 - By in vitro synthesis (siRNA, long RNAs)
 - By chemical synthesis (siRNA, modified oligos)

Establish QC at each step on the path to recording data?

Sequence verification

Feature quality

Sample isolation

RNA extraction

RNA stability

RNA amplification

Labeling

Hybridization

Washing

Scanning

“Just do the &%\$# array!”

Why QC and introduce standards?

Researchers have objective views about what a “good array” looks like.

There are multiple platforms.

Subtle changes can be buried in variation.

What is quality RNA?

Looks good on a Bioanalyzer trace?

Looks good on a gel?

Labels fine?

Amplifies?

Real-time PCR looks good?

Doesn't contain DNA?

Absorbs at 260 nm?

It is in our freezer and it took a year to get!

What tools and reagents can we make to both understand and limit variation?

Oh yeah, they have to be really easy to use and manufacture, marketable, inexpensive... and make us some money.

Sample collection

RNA isolation

RNA stabilization

RNA integrity analysis

RNA amplification and labeling

RNA/DNA hybridization

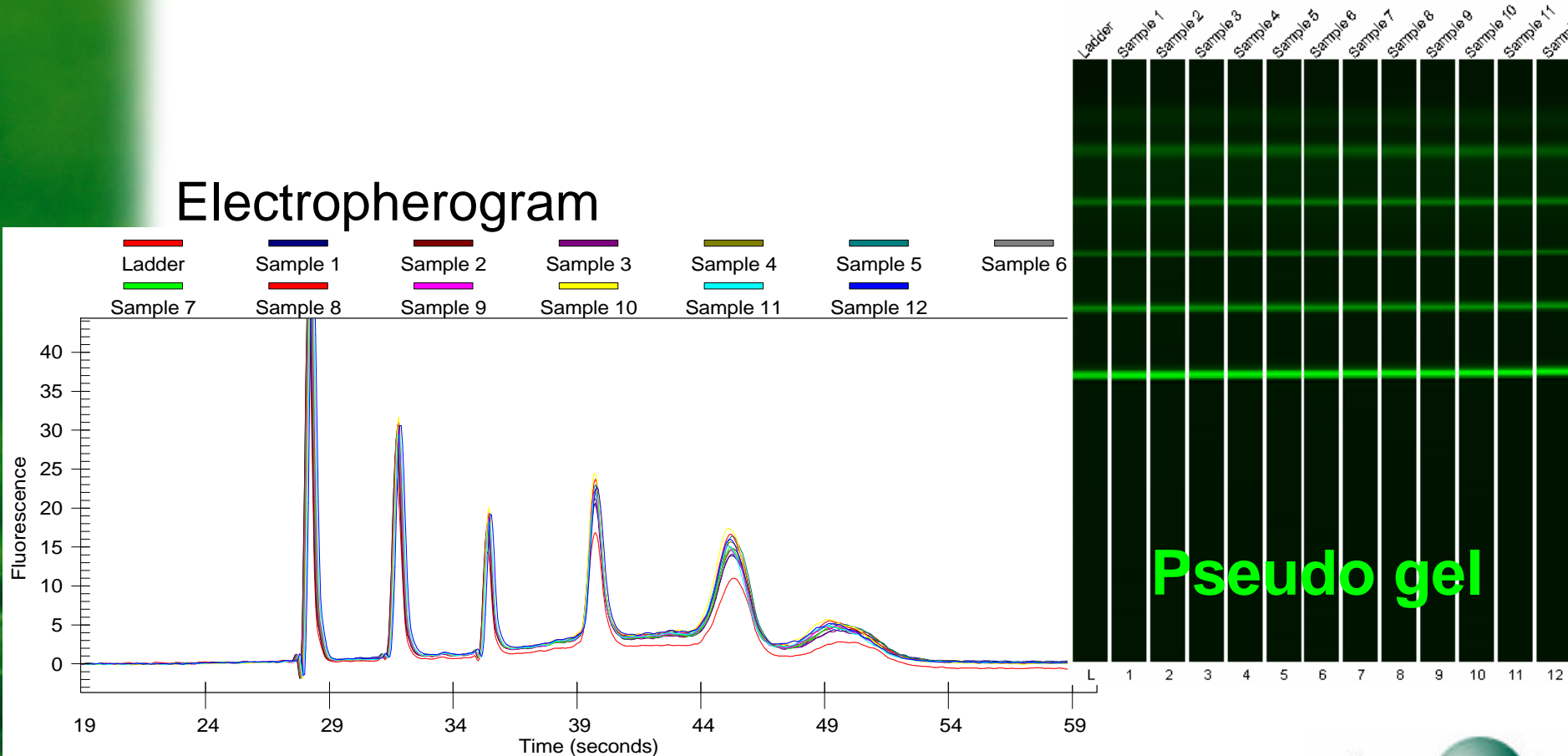
Array processing

Controls at each step

2100 Bioanalyzer is a must for RNA folks

12 samples of RNA ladder compared on one LabChip

Electropherogram



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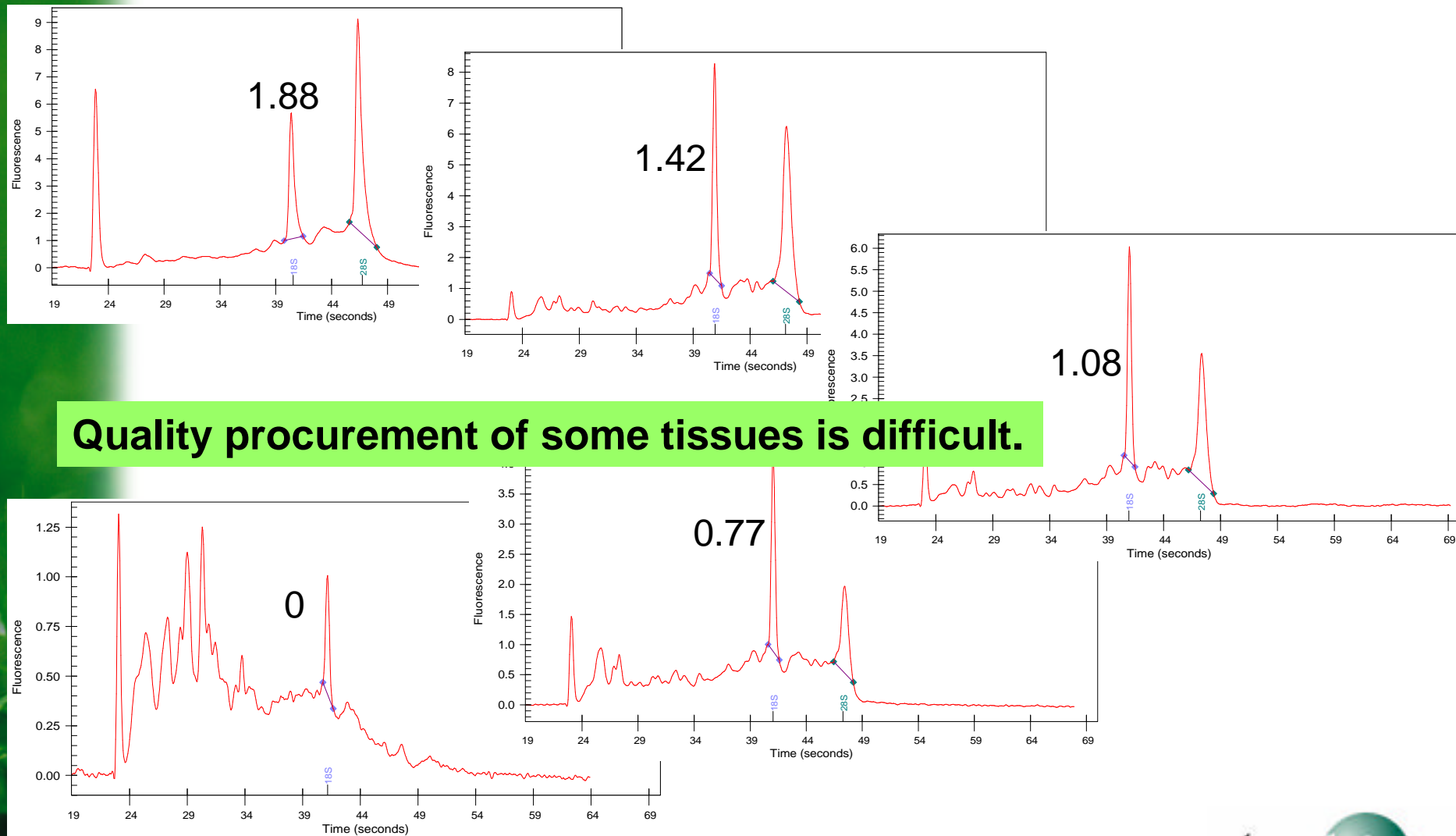
All RNA is Not Created Equal

**Procurement
Source
Tissue
Isolation method
Storage**

Tissue procurement is probably the most uncontrolled and variable step in the pathway to microarray data collection

Not sure how we can control this. Requires influencing the collection process. Future tools need to be easy with alternative methods available in order to minimize degradation.

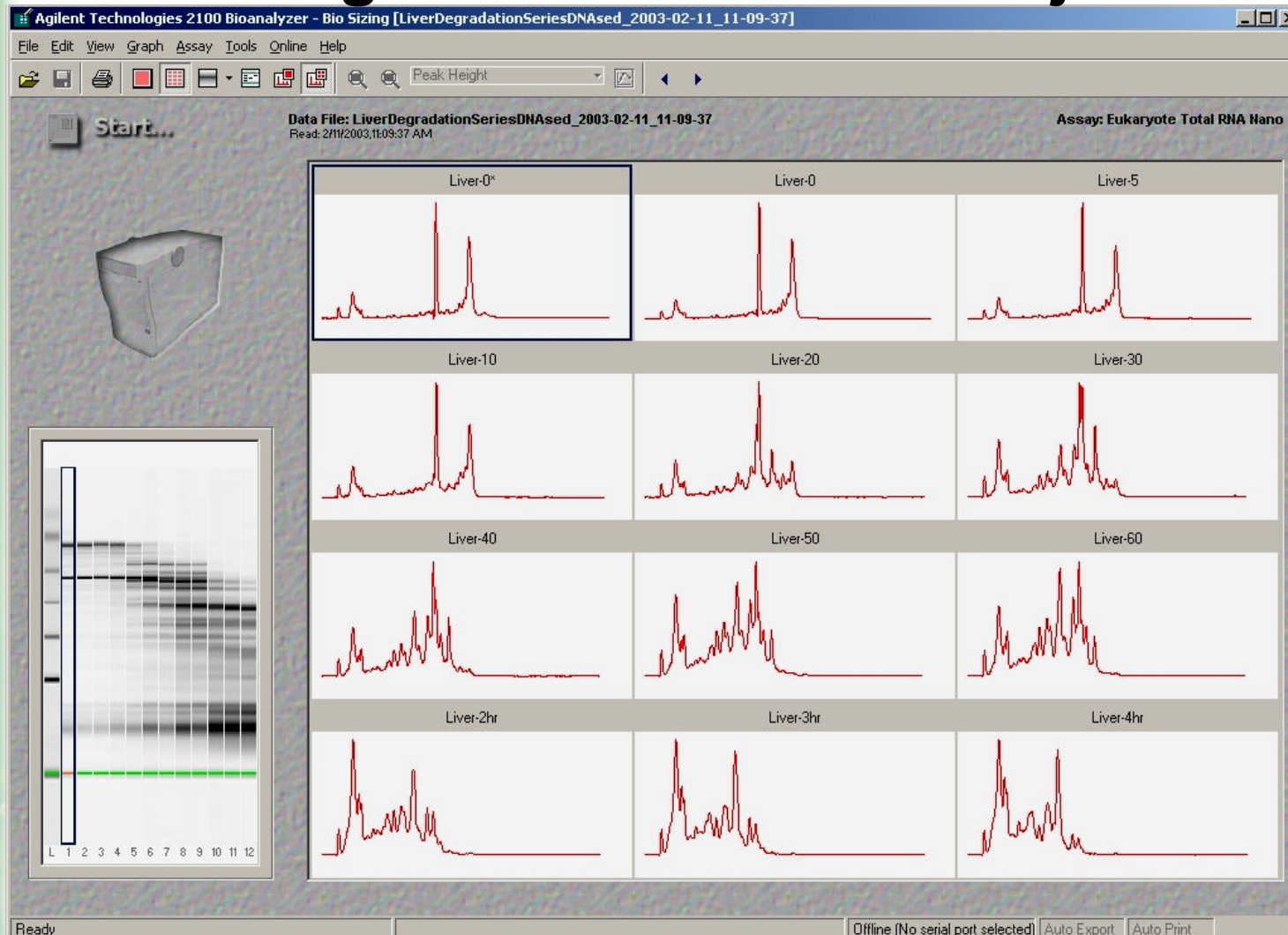
Five Prostate RNAs- same RNA isolation method



Quality procurement of some tissues is difficult.

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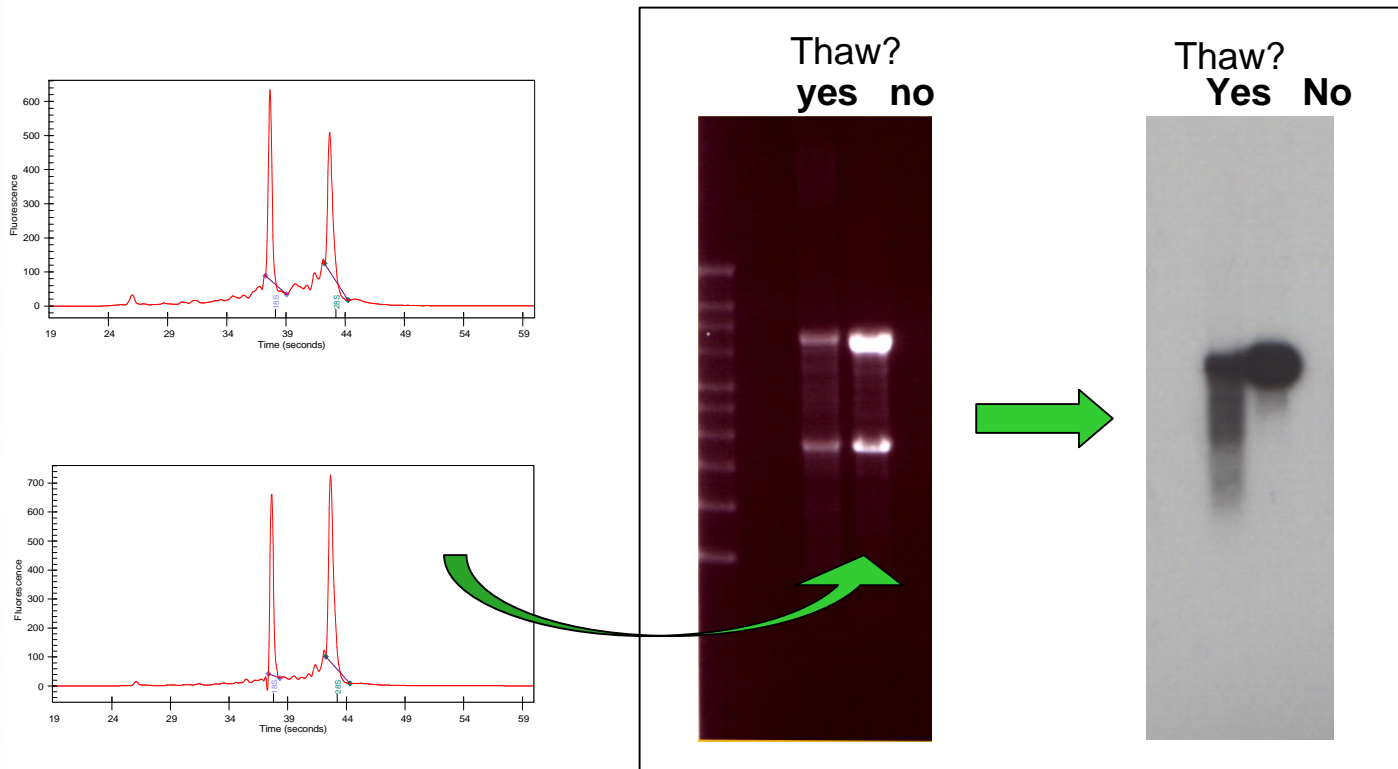
RNA degrades fast in it's own juices.



Liver sitting on the bench for the indicated mins and hours prior to RNA purification

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Effects of tissue freeze/thaw on RNA quality



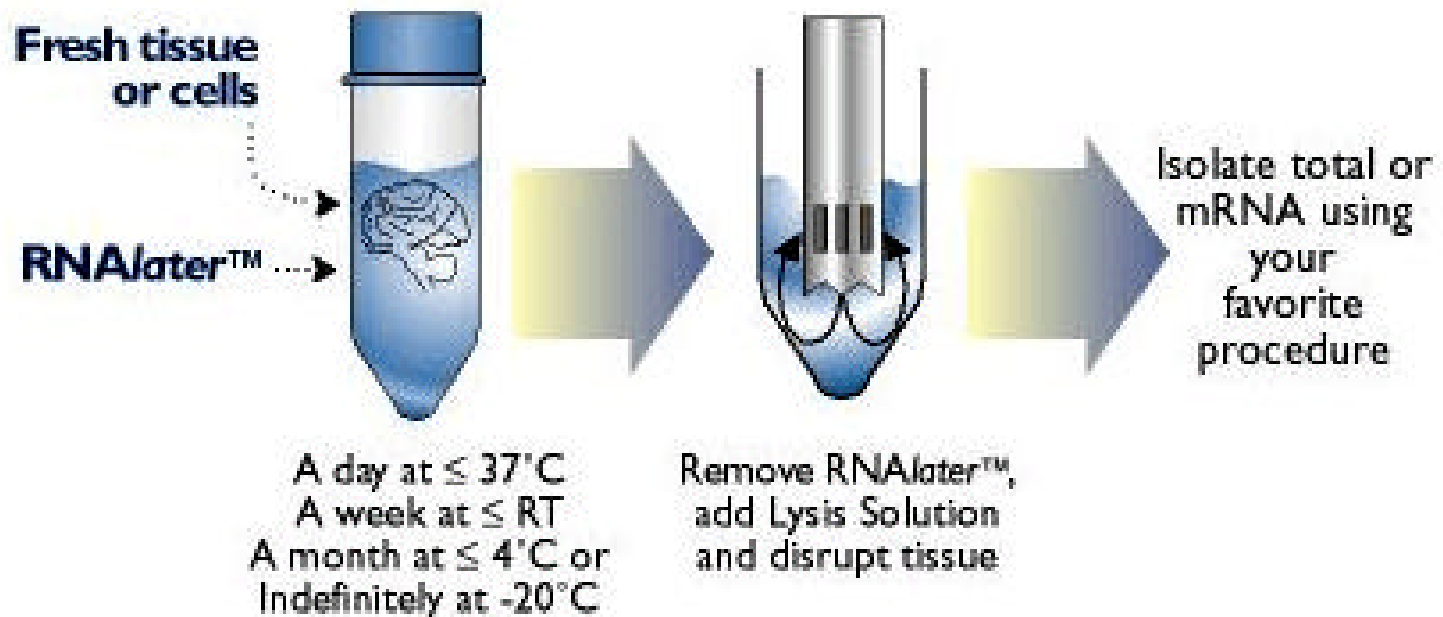
Agilent 2100 Bioanalyzer

EtBr Agarose b-actin Northern
(equal mass loading on gel)

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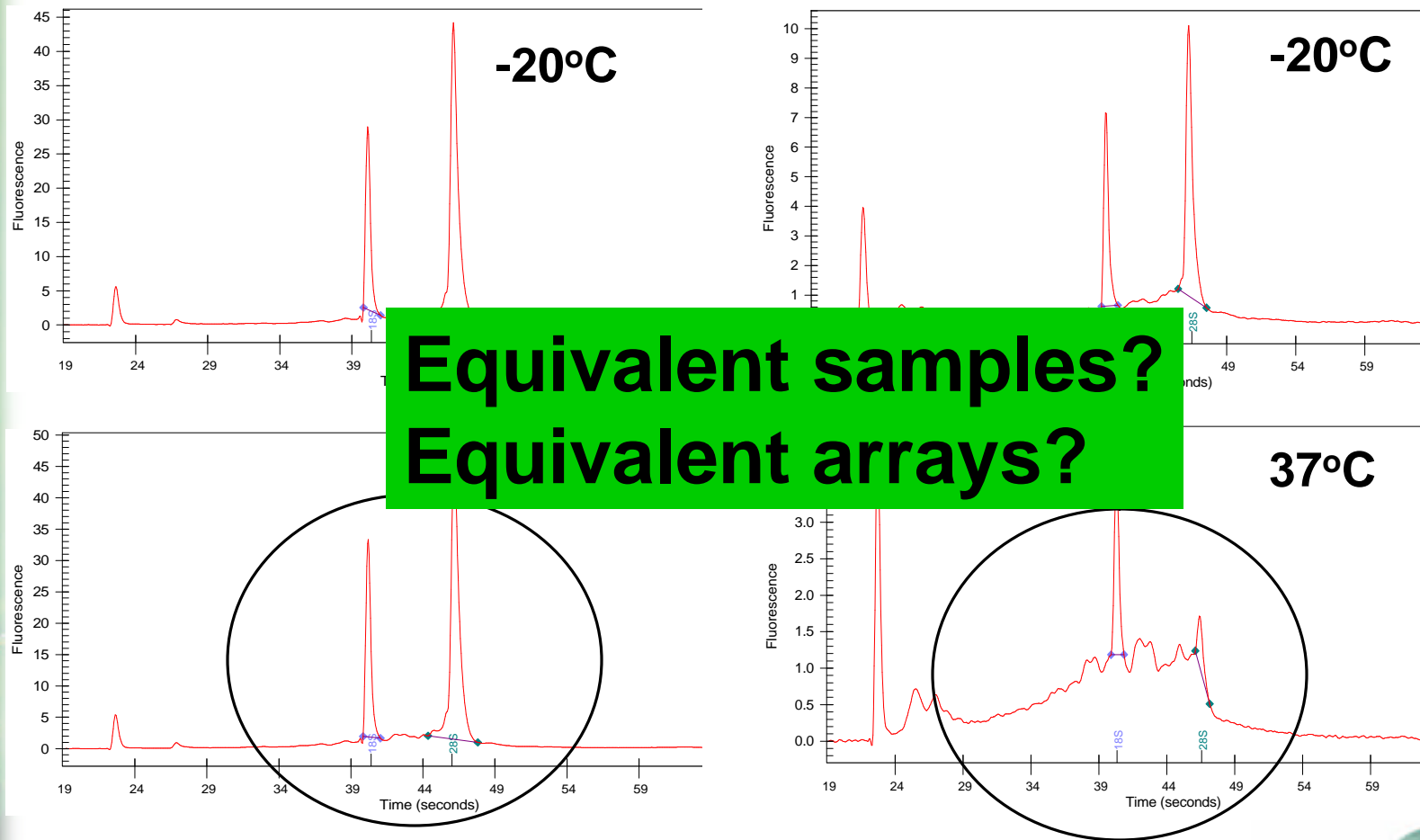
RNA Later Tissue Storage

N₂, ice, immediate lysis ?



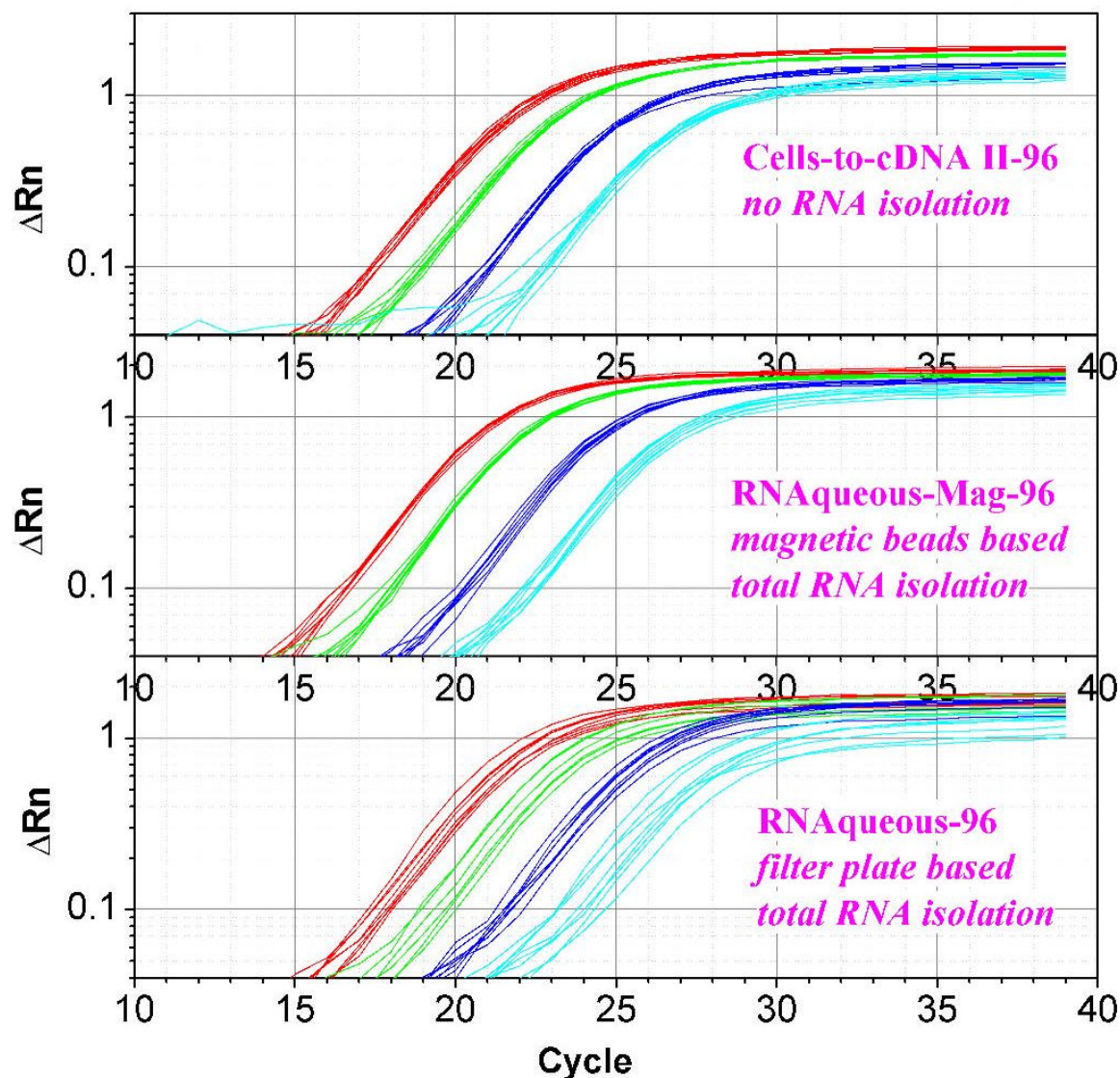
Isolating RNA: Stability?

Variable Stability of Prepared RNA



RNA Isolation can alter downstream applications

HeLa cells: 100,000, 20,000, 4,000, 800 per well. 5% RNA is used for qRT-PCR. Rho A was probed.

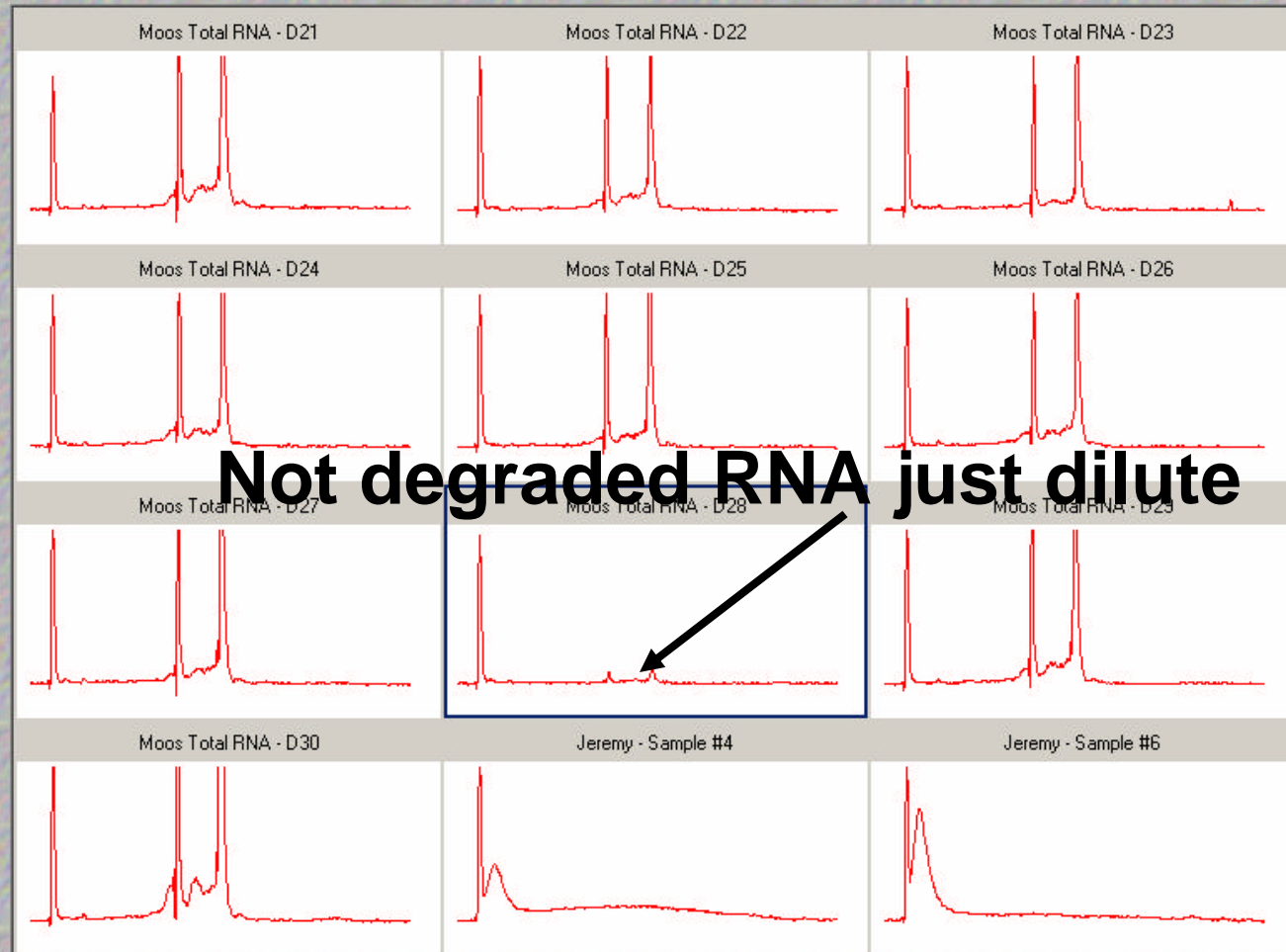
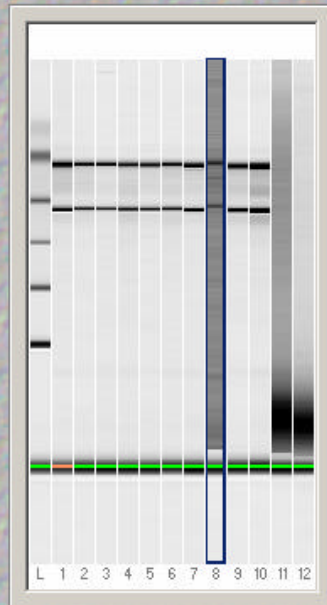


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Start...

Data File: 10-31-02 Moos total RNA D21-D30
Read: 10/31/2002, 11:29:41 AM

Assay: Eukaryote Total RNA Nano



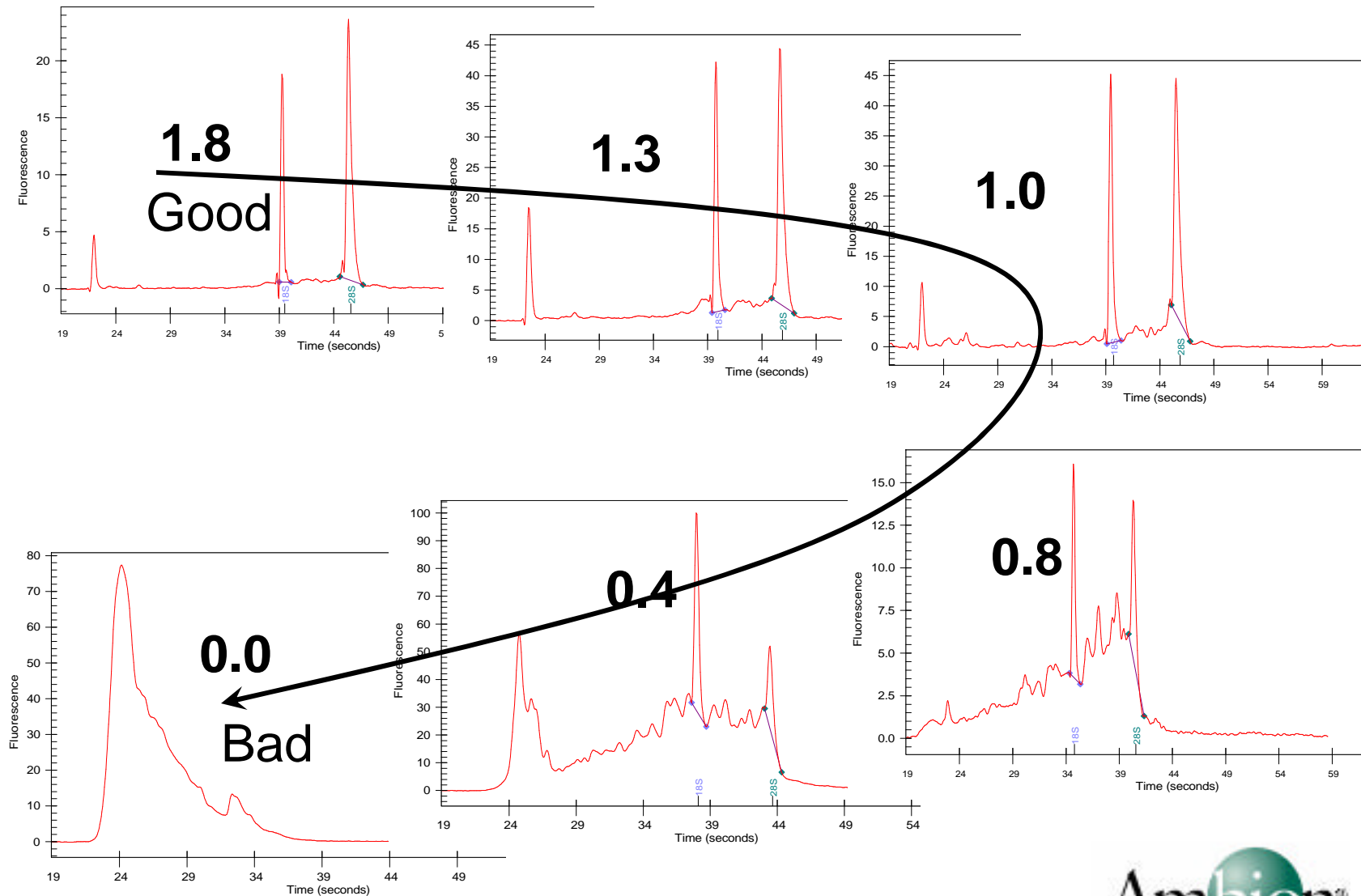
Ready

Offline (No serial port selected)

Auto Export

Auto Print

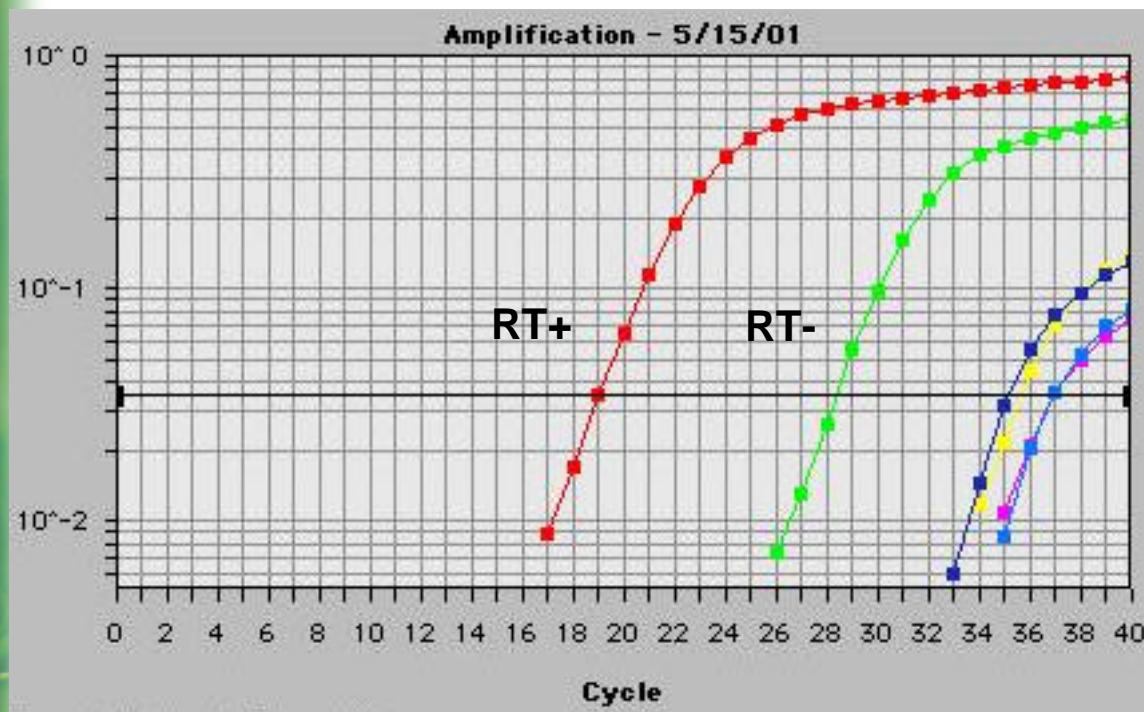
RNA Quality Index for Microarray Standardization



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DNase I treatment of RNA

This sample starts out at 4% DNA



| <u>DNase I/time</u> | <u>fold reduction</u> |
|---------------------|-----------------------|
| RT+ | - |
| 0 | 0 |
| 1X/30' | ~100 |
| 1X/60' | ~250 |
| 2X/30' | ~250 |
| 4X/15' | ~100 |

99.6% removal
(0.016% DNA)

G3PDH TaqMan assay

1X DNase = 0.02 units/ul

RNA Quality Index

- Sample isolated with minimal degradation
- • RNA extracted using the same method
- • RNA is proven stable during study
- RNA has a 28S:18S ratio above 1.5
- Free of DNA for Real-time validation

The procedures are available but not all tools are easily used or in simple kit formats.

What about small samples from LCM, tumors and preserved fixed tissues?

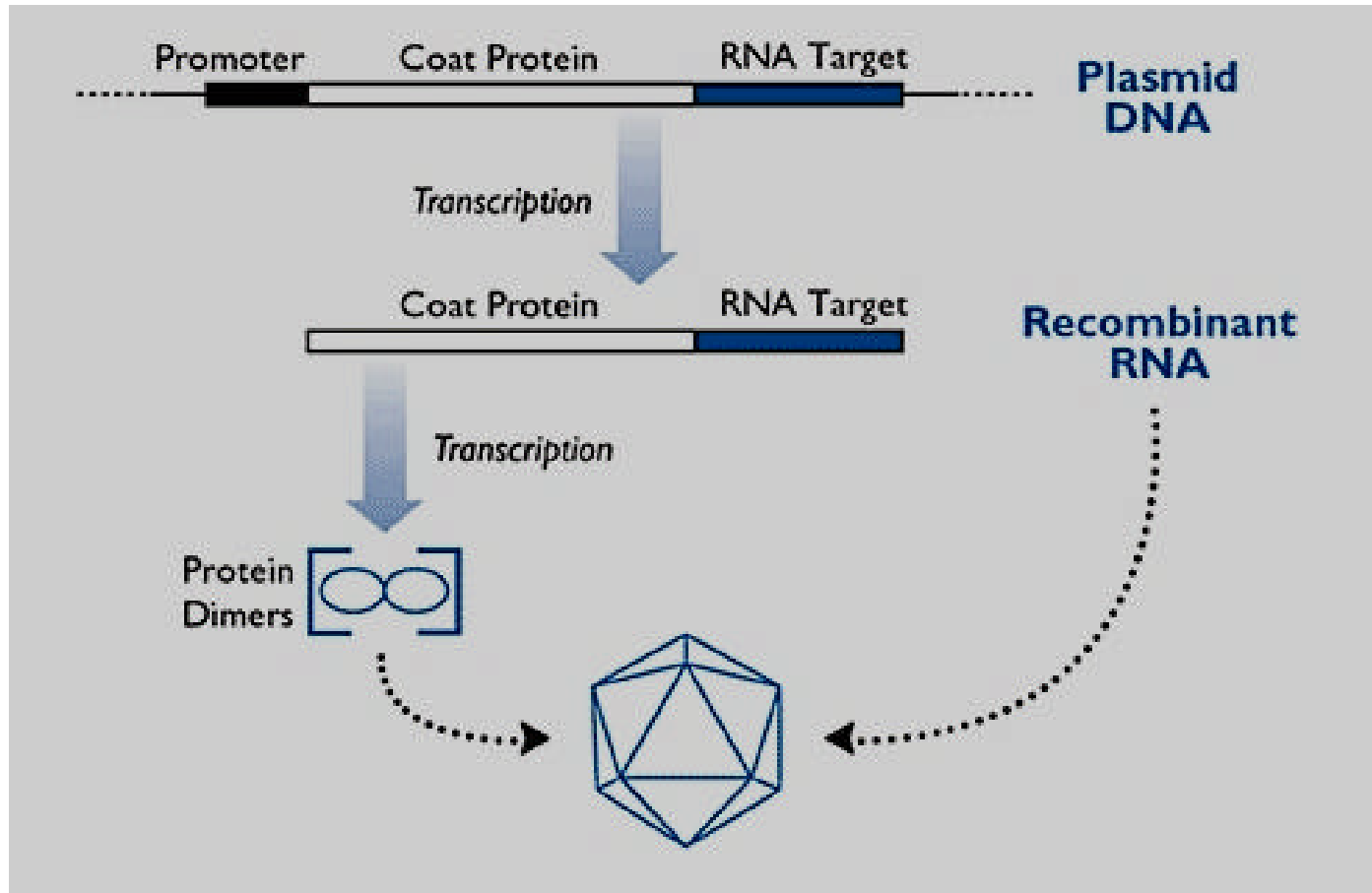
No excuse for the lack of universal spiked-in controls

- Affymetrix GeneChip has features for spiked in controls
- Amersham CodeLink has features for spiked in controls
- Agilent microarrays have features for spiked in controls
- Commercial Oligo sets can easily add control sequences
- Several companies offer control sets

Will universal set improve future analysis?

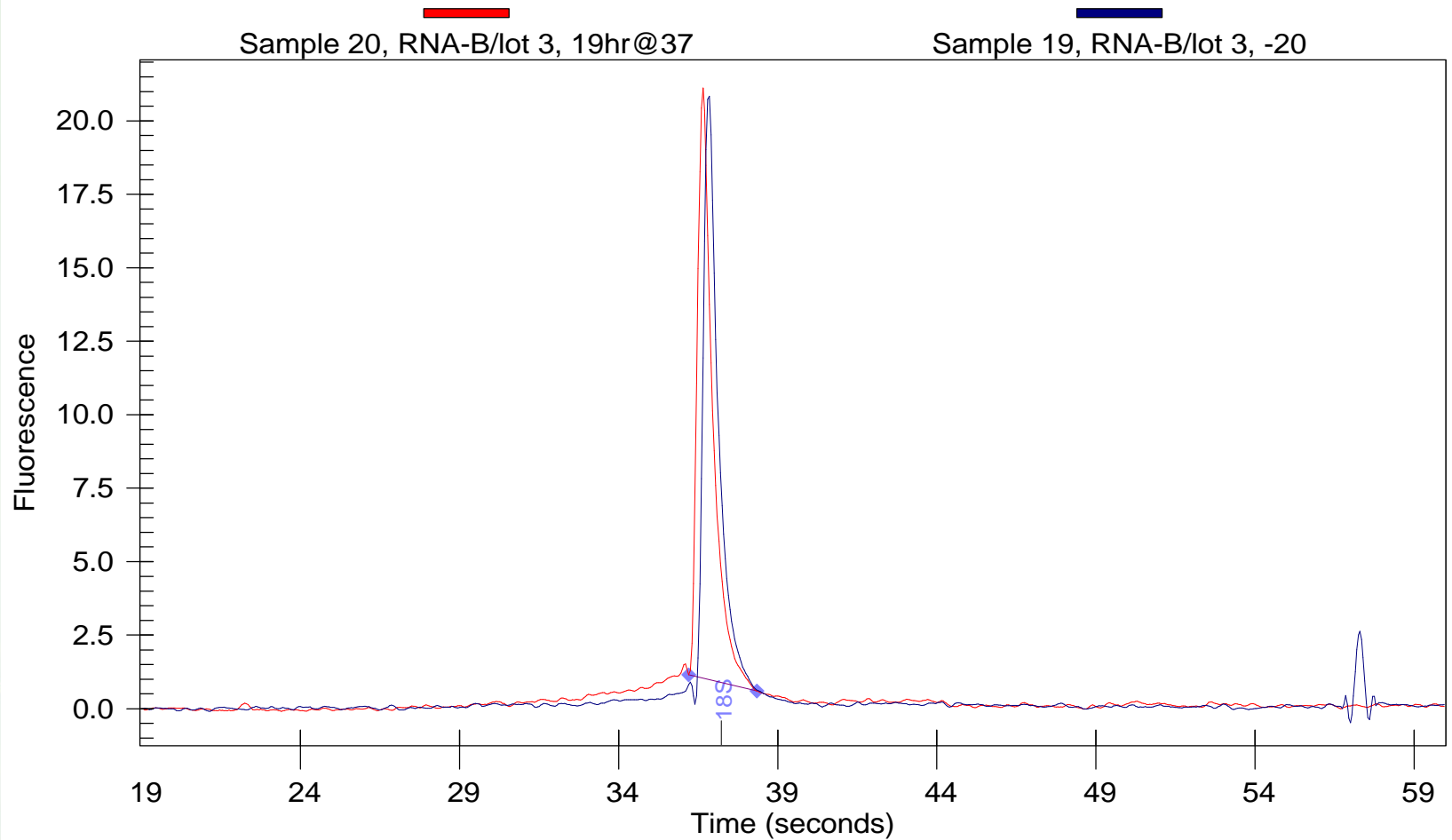
Ribonuclease resistant standards and controls

Armored RNA



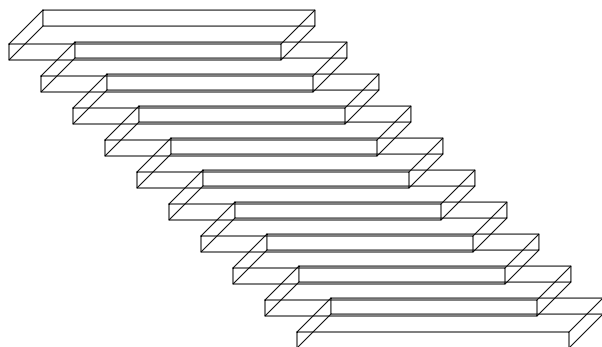


RNA transcripts can be easily and accurately QC'd



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Large Scale Robotic Production of RNA



96 well plates of PCR templates

Pool plates ?

T7 Transcription

Purify, quantify and QC

Mix and QC on arrays

Two runs each plate

(10ug each PCR template)

(2 mg each RNA)

Rough estimates:

2×10^9 pg each RNA
-might need about 20 pg/spot/array
= $\sim 1 \times 10^8$ units of standards
@1000 units per vial
100,000 vials

Other Reference Possibilities

Labeled feature specific oligonucleotides

Large scale produced-Labeled Genomic DNA

PCR generated fragments (DNA and RNA)

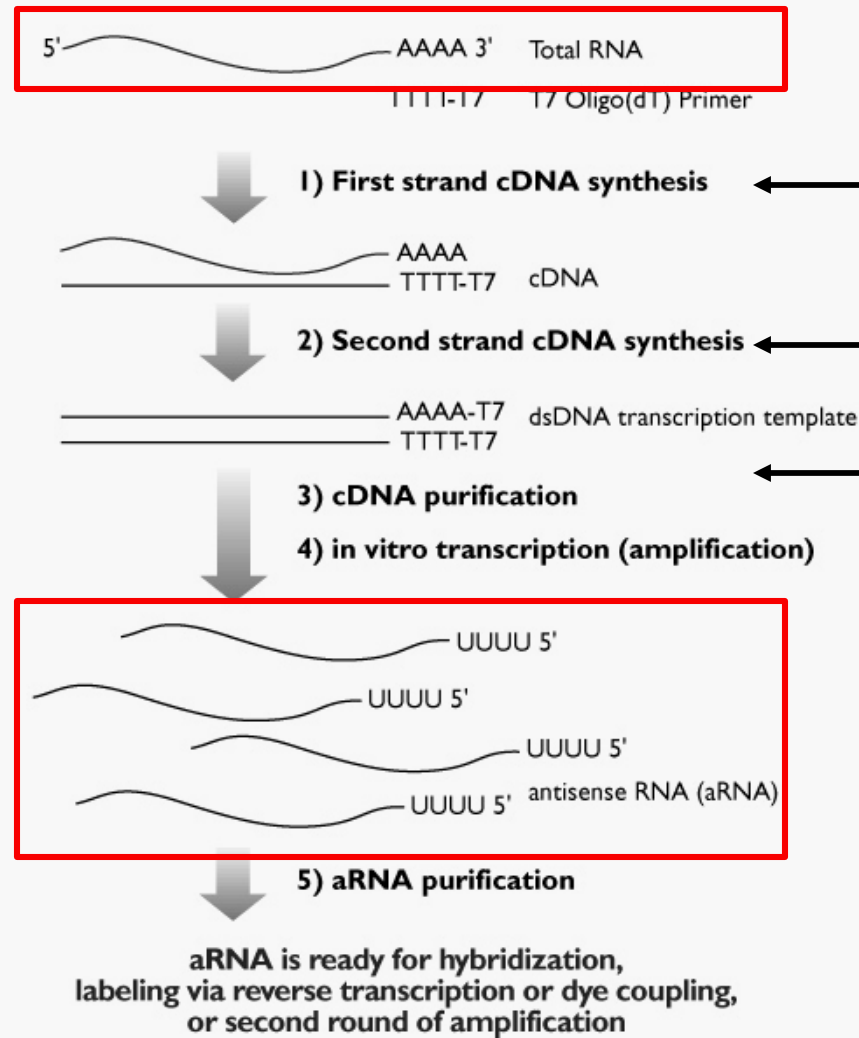
Pooled tissues and cell cultures

Whole body RNA

**Do we need to eliminate the
very abundant mRNAs?**

**Is there an optimal concentration of RNA?
Can we make one vial of standards that can
Be diluted to appropriate concentration depending
On the array platform used.**

RNA Amplification



These steps are generally ignored

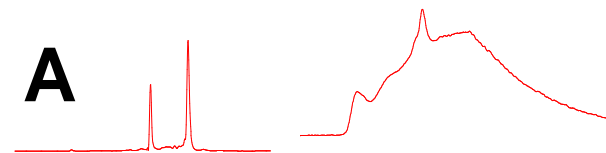
Realtime PCR Assays using the poly A controls

Qualifying RNA samples for further use in a microarray experiment

Total RNA

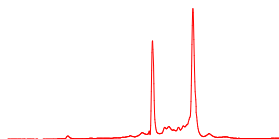
aRNA

A

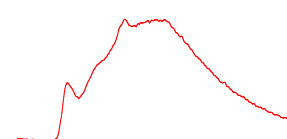


Total RNA sample 154
Glass filter 951.93ng/ul

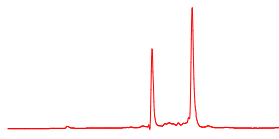
aRNA sample 154
759.46ng/ul (76ug)



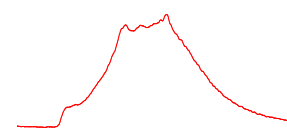
Total RNA sample 156
Glass filter 585.13ng/ul



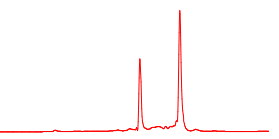
aRNA sample 156
663.51ng/ul (66ug)



Total RNA sample 158
Glass Filter 574.68ng/ul



aRNA sample 158
672.45ng/ul (67ug)



Total RNA sample 162
Glass Filter 862.91ng/ul

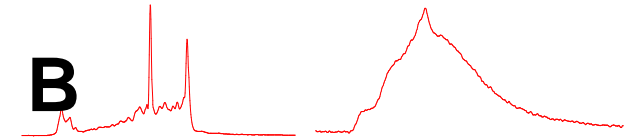


aRNA sample 162
871.03ng/ul (87ug)

Total RNA

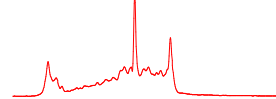
aRNA

B



Total RNA sample 140
Phenol 369.22ng/ul

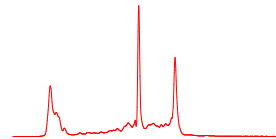
aRNA sample 140
230.82ng/ul (23ug)



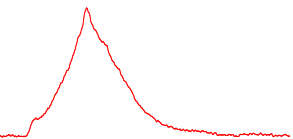
Total RNA sample 117
Phenol 689.08ng/ul



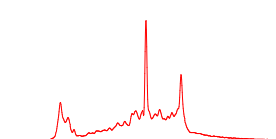
aRNA sample 117
260.51ng/ul (26ug)



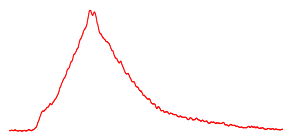
Total RNA sample 127
Phenol 726.21ng/ul



aRNA sample 127
100.06ng/ul (10ug)



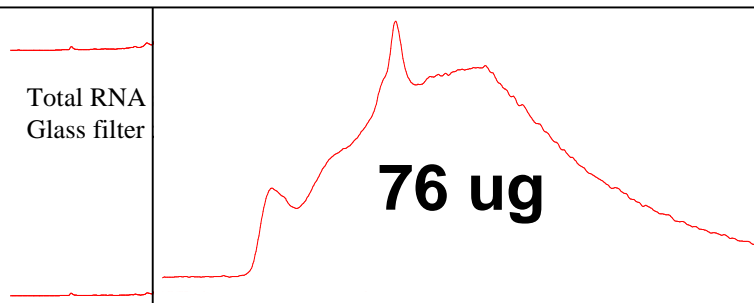
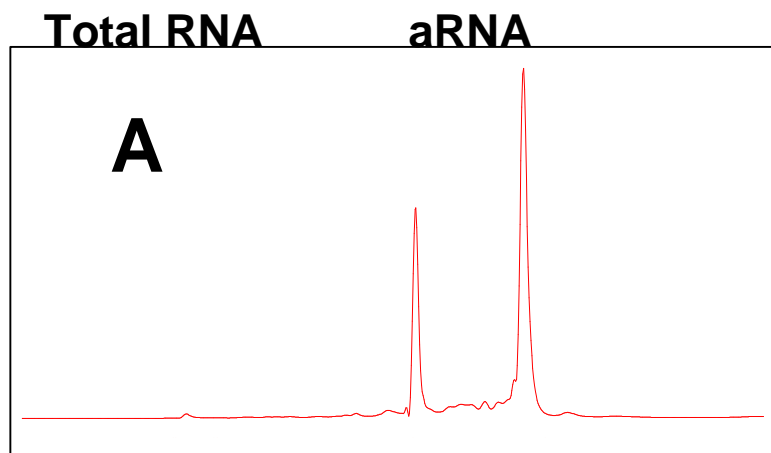
Total RNA sample 104
Phenol 339.44ng/ul



aRNA sample 104
129.73ng/ul (13ug)

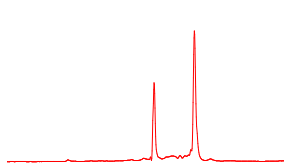
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Qualifying RNA samples for further use in a microarray experiment

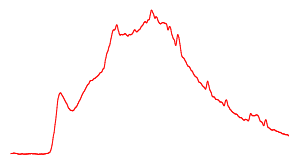


Total RNA sample 158
Glass Filter 574.68ng/ul

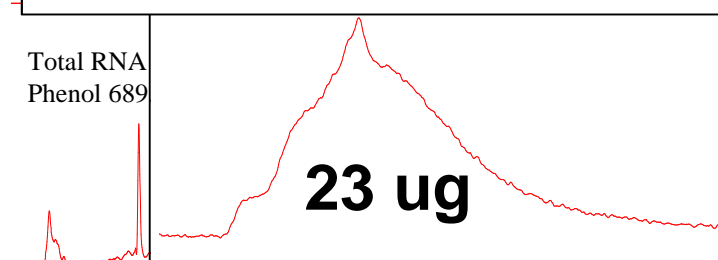
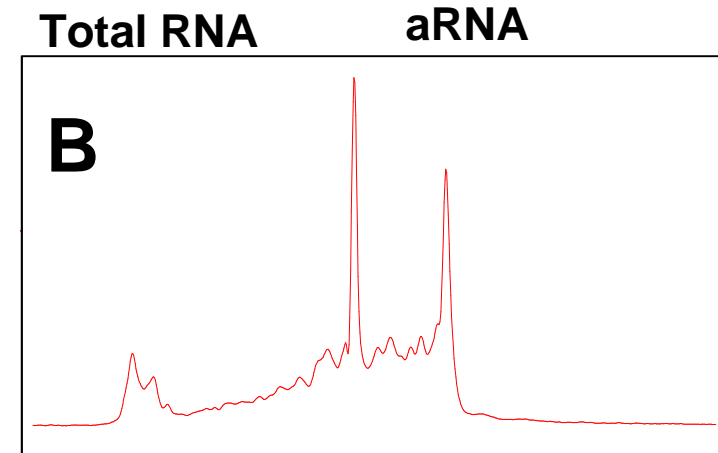
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Glass Filter 862.91ng/ul

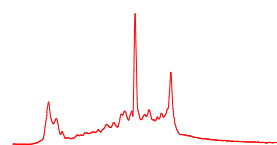


aRNA sample 162
871.03ng/ul (87ug)

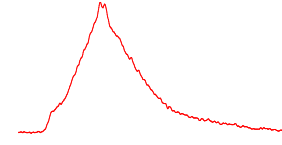


Total RNA sample 127
Phenol 726.21ng/ul

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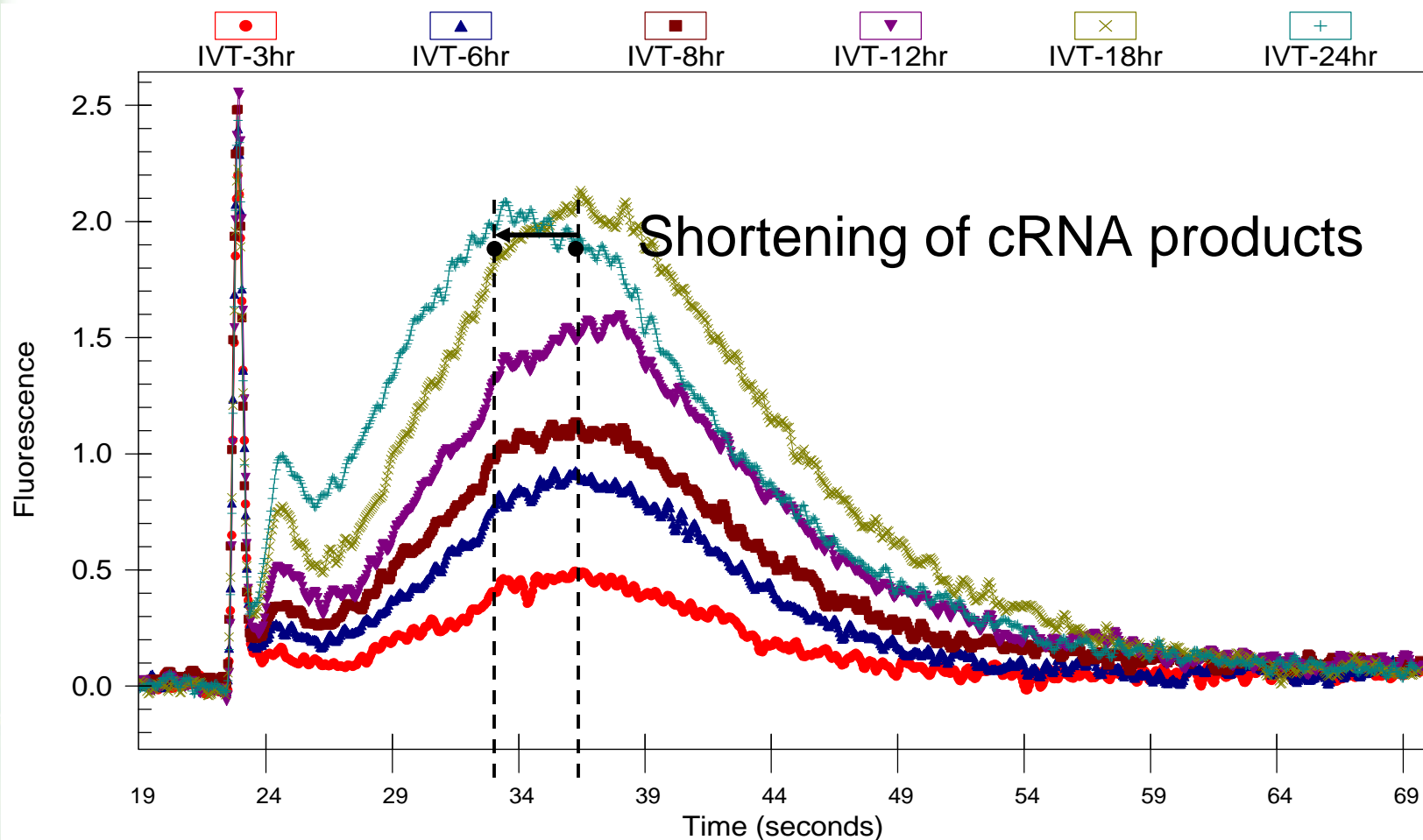
Total RNA sample 104
Phenol 339.44ng/ul



aRNA sample 104
129.73ng/ul (13ug)

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Observing subtle aRNA profile changes



What are the real effects on array quality?

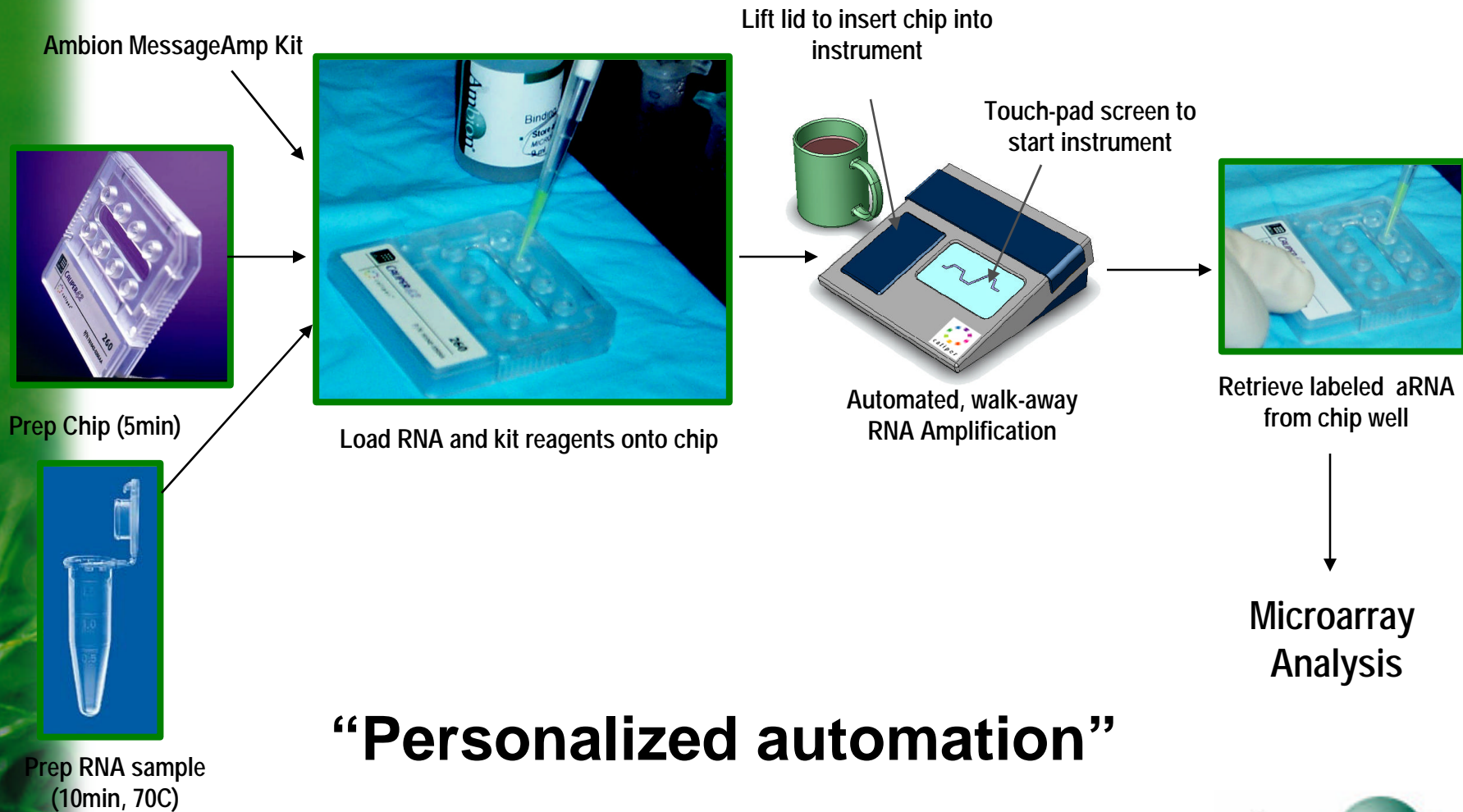
Physically control the Standardization using Automated process- Microfluidics

Started a microfluidic program with Caliper Technologies



Goal: use the benefits of automation and microfluidic properties to create much improved RNA research products on chips

Microfluidics Project initiated for RNA Amplification



“Personalized automation”

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Conclusions:

- The gross and subtle effects of RNA quality need to be studied on several array platforms (publications). **Control may not be possible but effects could be defined.**
- An RNA Quality Index can be formulated.
- Commercial kits to derive the RQI value may provide incentive for researchers to use and benefit databases.
- Mix and match approaches to sample processing may be creating “dirty databases”.
- Integrated automated solutions for RNA processing may eliminate much of the handling variability across the thousands of array labs.
- Human samples- Collection is the critical point of RNA quality and don't forget the state of the donor at collection.

Contributing Members

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